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HEMATOLOGY

Effect of Freezing and Thawing on Uptake of Amino Acids into Human Erythrocytes

DR. RICHARD MOORE*, DR. RITA PARENTI**, DR. I. RÉNA BIZIOS***

ABSTRACT — The transport of ^{14}C-amino acids into human erythrocytes and into erythrocytes which had been frozen and thawed was studied experimentally. The relative rates of uptake were found to be (in decreasing order): phenylalanine, leucine, alanine, trythophan, and glycine. Freezing (using gycerol as the cryoprotective additive) and thawing had no significant effect on either the magnitude of the rate constants or on the order of the above relative rates of amino acid uptake into erythrocytes suspended in isotonic TRIS-HC1 buffer.

The preservation of cells and tissues by freezing is becoming a method of increasing importance and frequent use in bacteriology, obstetrics, surgery, hematology, and animal husbandry. Various procedures and instruments have been proposed and/or employed to process cells or tissues for long-term preservation. It is necessary and important, therefore, to evaluate these procedures and instruments.

Freezing and thawing are assumed to affect the normal physiology of the red blood cells and to damage the cell membrane. Membrane transport studies provide a way for detecting and measuring the extent and the effect of such damages. In this respect, Wallach, *et al.*, (1962) reported that frozen-deglycerolized human erythrocytes retain their normal cation flux and electrolyte concentration.

There are no results reported in connection with the amino acid uptake into frozen-thawed red cells. The transport of neutral amino acids into human erythrocytes, however, has been the subject of numerous experimental studies. A summary of the most important work before 1964 is given by Winter and Christensen (1964).

The uptake rates and steady state accumulation of ^{14}C-amino acids under various conditions have been investigated and compared. Winter (1962) and Winter and Christensen (1962) studied transport into erythrocytes suspended either in plasma of heparinized blood or in Raker's medium. Their results distinguished between initial rates of entry and steady state intracellular amino-acid-accumulation. Contrary to the following order of initial rate of entry L-leucine > . . . . > L-alanine > . . . . >glycine, and after a long time of incubation, glycine showed the highest intracellular accumulation, for example. Furthermore, Winter and Christensen reported a direct relation between transport rates and the size of the apolar hydrocarbon side chain of the entering amino acid. In terms of kinetics they classified three modes of uptake: a mediated and easily saturable type for long chain amino acids; a second nonsaturable type for amino acids with large hydrocarbon side chains; and a third, low-capacity, saturable type for alanine and glycine. Fast counter-transport was observed for the amino acids that use the leucine-type transport system. Comparable results were reported by Oxender (1962) in connection with the amino acid transport into Ehrlich ascites tumor cells.

Björnesjö (1964) studied (what appears to be steady state) accumulation of amino acids in erythrocytes suspended either in plasma or in TRIS-HC1 buffer. His results showed high uptake for glycine, phenylalanine, and lysine, intermediate for tryptophan, and negligible uptake for glutamic acid.

A comparative experimental study was conducted to determine the effect (if any) of freezing and thawing on the amino acid transport into erythrocytes.

Materials and methods of collecting

The ^{14}C-amino acids used in this study were obtained in a highly purified grade from the New England Nuclear Corporation (Boston, Massachusetts).

Human blood was collected from healthy adult donors in polyethylene bags containing 75 ml. of citric acid-sodium citrate-dextrose anticoagulant per 445 ml. of venous blood. The erythrocytes were separated qualitatively by centrifugation (5,000 x g for 10 minutes). Several aliquots of packed red cells were stored overnight at 4 C, while several others were mixed with glycerol (15 percent by volume as a cryoprotective agent; Pert, *et al.*, 1963; 1965), and were frozen and stored overnight at −170 C.

Samples of the erythrocytes stored at 4 C were washed with isotonic TRIS-HC1 buffer (pH 7.6) containing 0.1 M NaC1, 0.039M KC1, 0.005M $MgC1_2$, 0.001M phosphate buffer, and 0.044M glucose. After three washings the packed erythrocytes were suspended in isotonic TRIS-HC1 buffer solution (final pH 7.4; haematocrit

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20-25%; haemoglobin 10gm/100 ml.) containing 0.2 mM ^{14}C-amino acid.

The ^{14}C-amino acid-erythrocyte suspension was incubated at 37 C in a Dubnoff Shaking Incubator (New Brunswick Scientific Corporation) with continuous, gentle (90 rpm) shaking to assure uniform mixing. Samples (10 ml.) were withdrawn at various time intervals (3 min., 30 min., 1, 2, 3, and 4 hours) during the incubation. "Separaid" polystyrene beads (Unitech Chemical Manufacturing Company) were added, and the samples were centrifuged at 1,200 x g for 10 minutes. A 500 μl. sample of the supernatant medium, and a weighted 0.5 ml. sample of the packed erythrocytes were taken.

The ^{14}C-amino-acid-containing erythrocytes were lysed in 1 ml. of distilled water and the proteins were precipitated with the addition of 3.5 ml. of 10 percent cold trichloracetic acid. The sample was kept in an ice bath for 30 minutes, was centrifuged, and then 0.5 ml aliquots of the ^{14}C-amino-acid-containing supernatant were transferred into low-potassium-glass vials each one containing 15 ml. of fluor (1,000 ml. of dioxane, 100 gm. of naphthalene, 7 gm. of PPO and 50 mg. of POPOP). The 500 μl. samples of the supernatant medium were treated in a similar way.

All samples were analyzed for radioactivity with a dual channel beta spectrometer (Packard Tri-Carb Model 3203). Each sample was counted until two successive counts agreed within 3%.

At least four or five runs were made for each amino acid under investigation.

The frozen erythrocytes were thawed in warm water (45 C) and were washed once with isotonic normal saline-sucrose (30 percent by weight), and once with isotonic normal saline-mannitol (5 percent by weight) solutions. The procedure described in connection with the samples of erythrocytes stored at 4 C was then repeated.

Treatment of Data

The data obtained were two complimentary sets of counts of radioactivity (intracellular and extracellular) in terms of concentration of ^{14}C-amino acid per gram of sample. These values were corrected (by subtraction) for the counting rate of the background. Further corrections were made for: the plasma trapped between the packed cells; the non-solvent volume of the cells; cell density; and cell volume changes taking place during the experiments. The final values were averaged for the four or five experimental runs for each amino acid.

In analyzing the data, the following graphs were plotted: (i) radioassay values versus time of incubation; (ii) logarithm of the difference between the intracellular concentration of each amino-acid at a given time during incubation and at infinite time (log x) versus time; (iii) Δx versus time; and (iv) reciprocal Δx versus time. A Lineweaver-Burk plot also was attempted.

The relative rates of ^{14}C-amino-acid uptake into erythrocytes were found to be (in decreasing order): phenylalanine, leucine, alanine, trytophan, glycin. The freeze-thaw process used had no significant effect on either the above order or on the magnitude of the rate constants of the rate constants of the amino acid uptake (Table I).

The graphs of radioassay values versus time of incubation gave curves which appeared to be monotonic functions with a single exponential term.

The half-times required to reach the equilibrium concentration were determined graphically. The half-time values obtained from intracellular and extracellular radioactivity data were very close together. The rate of intracellular radioactivity increase, though, was more accurate because in that case the radioactivity varied from an initial zero concentration to some finite value (at the end of the incubation interval), instead of being a difference between two large numbers.

The graphical analysis of the data showed that the transport reaction could not be described by either zero or second order kinetics. Furthermore, it could not be described by a Michaelis-Menten equation. The reaction or, at least, the rate limiting reaction, appeared to be of first order. The calculated values of that rate constant (k) are given in Table I.

TABLE I — Correspondence between rates of entrance and amino acids into control or into frozen-thawed human erythrocytes.

Amino Acid	Half-time to reach steady state, minutes	
	Control*	Frozen-thawed
PHENYLALANINE	7	7
LEUCINE	26	12
ALANINE	35	35
TRYPTOPHAN	50	50
GLYCINE	145	120

*The control cells were non-frozen cells.

The results of amino acid uptake into erythrocytes and into frozen-thawed erythrocytes show that the freezing and thawing process used in the experiments did not disturb this aspect of cell physiology. These observations are in agreement with the work of Wallach, *et al.* (1962), who used a similar freezing-thawing method and found no change in the cation flux and electrolyte concentration of erythrocytes.

The order of the relative rates of amino-acid uptake is in good agreement with the order of entry rates reported by Winter and Christensen (1964).

Table II shows that the observed rate of transport decreased as the molecular weight of the respective amino acid increased. This observation supports the conclusions of Oxender, Winter, and Winter and Christensen in connection with the steric relationships involved in amino acid transport. Diffusion did not appear to be the primary

TABLE II — Comparison of the variation of the rate of entrance with the variation of the molecular weight of the amino acids.

Amino Acid	Half-time for control cells, minutes	Molecular Weight
PHENYLALANINE	7	165
LEUCINE	26	131
ALANINE	35	89
TRYPTOPHAN	50	204
GLYCINE	145	75

mode of transport in the present study; amino acid transport might thus be dependent on carriers in the cell membrane as proposed by Oxender, Christensen, and their associates.

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